Rottlerin, a Specific Inhibitor of Protein Kinase C-delta, Impedes Barrier Repair Response by Increasing Intracellular Free Calcium

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Several signals have been suggested in maintaining skin barrier homeostasis, but epidermal calcium ions are currently thought to be a main signaling factor. It is not clear, however, exactly how an intracellular calcium level decreases in response to the loss of an extracellular calcium gradient. In this study, we investigated the effects of several broad-type and isozyme-specific protein kinase C (PKC) inhibitors on epidermal permeability barrier recovery. Topical application of chelerythrine chloride, a broad-type PKC inhibitor, and rottlerin, a PKCδ-specific inhibitor, significantly impeded the barrier recovery rate at 3 and 6 hours after barrier disruption. A significant decrease in the number and secretion of lamellar bodies was also observed at the inhibitor-treated site. Calcium ion-capture cytochemistry showed that the epidermal calcium gradient was rapidly reformed in inhibitor-treated skin, though recovery of the corresponding barrier function was not observed. In cultured keratinocytes treated with either inhibitor, increased intracellular calcium did not return to the baseline concentration after extracellular calcium decreased. These results suggest that PKC inhibitors, especially a PKCδ-specific inhibitor, delay barrier recovery by affecting the intracellular calcium concentration after a loss of the extracellular calcium gradient. Furthermore, PKCδ is important in controlling a decrease in intracellular calcium concentration.

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INTRODUCTION

The outer sheath of the skin, the stratum corneum (SC), not only defends against the entry of microorganisms but also protects against excessive water loss. Disruption of this permeability barrier has been reported to occur in several inflammatory dermatoses, including allergic contact dermatitis (Hachem *et al.*, 2001; Held *et al.*, 2001), hand dermatitis (Agner, 1992), hypertrophic scar and keloid (Suetake *et al.*, 1996), atopic dermatitis (Werner and Lindberg, 1985; Berardesca *et al.*, 1990; Watanabe *et al.*, 1991; Loden *et al.*, 1992), and psoriasis (Tagami and Yoshikuni, 1985; Ghadially *et al.*, 1996). The permeability barrier can also be artificially disrupted by topical treatment with organic solvents, tape stripping, or detergents (Feingold, 1997).

Regardless of the manner of barrier disruption, the barrier repair response leads to a restoration of normal barrier function. Immediately after barrier disruption, all of the lamellar bodies (LBs) in the outermost granular cells are secreted (Menon *et al.*, 1992b), and an increase in both cholesterol and fatty acid synthesis occurs. In contrast, a delayed increase in ceramide synthesis (Holleran *et al.*, 1991) as well as a stimulation of epidermal β-glucocerebrosidase (Holleran *et al.*, 1994) and DNA synthesis (Proksch *et al.*, 1991) are associated with the late phase of barrier recovery. To address this issue, it is crucial to know the intracellular signals involved in inducing the above-mentioned biological responses after cutaneous barrier disruption.

Calcium ions act as a major regulator in keratinocyte differentiation and proliferation, and epidermal calcium ions are thought to be a main signaling factor in maintaining skin barrier homeostasis. It is unclear, however, what role losing the extracellular calcium gradient plays in the barrier recovery response after cutaneous barrier disruption. In addition, the mechanism by which the intracellular free calcium level concentration ($[Ca^{2+}]_i$) decreases in response to the loss of the extracellular calcium gradient is also unclear.

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Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium level; $[Ca^{2+}]_o$, extracellular free calcium level; LB, lamellar body; PKC, protein kinase C; SC, stratum corneum; SG, stratum granulosum; TEWL, transepidermal water loss

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We studied the roles of protein kinase C (PKC) inhibitors and $[Ca^{2+}]_i$ regulation in barrier repair after acute barrier disruption. By measuring transepidermal water loss (TEWL) and using morphological studies, we found reduced barrier recovery with PKC inhibition, particularly PKCδ-specific inhibition. The $[Ca^{2+}]_i$ measurements suggest that this reduced barrier recovery is due to a lack of intracellular calcium response to an extracellular calcium change.

RESULTS

Barrier recovery rate of broad-type PKC inhibitors

Repeated tape stripping was carried out on both sides of the flank of male hairless mice. Chelerythrine chloride, a broad-type PKC inhibitor solution of appropriate concentration, and vehicle were applied to the barrier-disrupted site for 10 minutes. The stripped and treated skin showed significantly delayed barrier recovery when compared to vehicle-treated skin at 3 and 6 hours. The percent TEWL recovery decreased in a dose-dependent manner (Figure 1a). Another broad-type PKC inhibitor, calphostin C, also significantly delayed barrier recovery at 3 and 6 hours (Figure 1b).

Barrier recovery rate of isoenzyme-specific PKC inhibitors

We performed additional barrier recovery rate experiments to identify which specific PKC inhibitor impeded the barrier recovery response. The stripped skin treated with rottlerin, a PKCδ-specific inhibitor, showed a significantly delayed barrier recovery as compared to vehicle-treated skin at 3 hours. The percent TEWL recovery of rottlerin-treated skin decreased in a dose-dependent manner (Figure 2a). Other PKC isoform-specific inhibitors, Gö6976 (PKCα) and Mry (PKC zeta; pseudosubstrate sequence from human PKCζ), did not show significantly delayed barrier recovery at 3 and 6 hours (Figure 2b).

Formation of SC lipid lamellae and secretion of LB

We used Nile red staining and quantitative electron microscopy to assess changes in LB secretion and lipid deposition in the SC. The vehicle-treated skin showed an intense red staining in the Malphigian layer and the SC at 3 and 6 hours (Figure 3v). The chelerythrine chloride-treated skin showed very weak staining in the epidermis at 3 hours after barrier disruption and delayed staining at 6 hours (Figure 3c). Rottlerin-treated skin showed staining similar to the chelerythrine chloride treatment (Figure 3r). Gö6976-treated skin showed an intense red staining in the epidermis, much like vehicle-treated skin (Figure 3g).

Although there was acute barrier disruption, electron micrographs showed that the secretion of LB between the SC and stratum granulosum (SG; secreted) was significantly decreased. Considerable numbers of LB remained in the upper layers of the SG (unsecreted) following chelerythrine chloride treatment (Figure 4c). Similar findings were seen in the rottlerin-treated skin. Rottlerin treatment also showed a decrease in the number of LBs between the SC and SG, and many LBs remained in the upper layers of the SG at 3 hours (Figure 4r). Treatment with the PKC-α-specific inhibitor, Gö6976, showed normal secretion of LB to the SC and fewer LBs remained within the SG (Figure 4g), similar to vehicle-only treatment (Figure 4v). At 30 minutes after barrier disruption, LB secretion between the SC and SG in chelerythrine chloride- and rottlerin-treated skin was significantly lower than that in vehicle-treated skin. Furthermore,

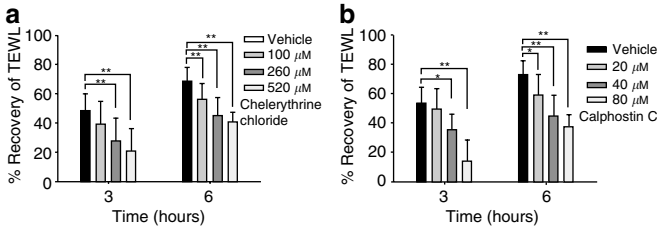

Figure 1. Broad-type PKC inhibitors delay the barrier recovery. (**a**) Topical application of chelerythrine chloride, a broad-type PKC inhibitor, delayed the barrier recovery rate in a dose-dependent manner. (**b**) Another broad-type PKC inhibitor, calphostin C, also significantly delayed barrier recovery at 3 and 6 hours (*$P<0.05$, **$P<0.01$).

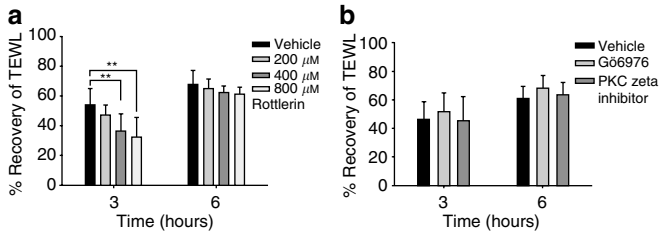

Figure 2. PKCδ-specific inhibitor delays the barrier recovery. The stripped skin treated with rottlerin showed significantly delayed barrier recovery as compared to vehicle-treated skin at 3 hours. (**a**) The percent TEWL recovery in rottlerin-treated skin was decreased in a dose-dependent manner. (**b**) Other PKC isoform-specific inhibitors, Gö6976 (PKCα) and Mry (PKC-zeta; pseudosubstrate sequence from human PKCζ), did not show significantly delayed barrier recovery at 3 and 6 hours (**$P<0.01$).

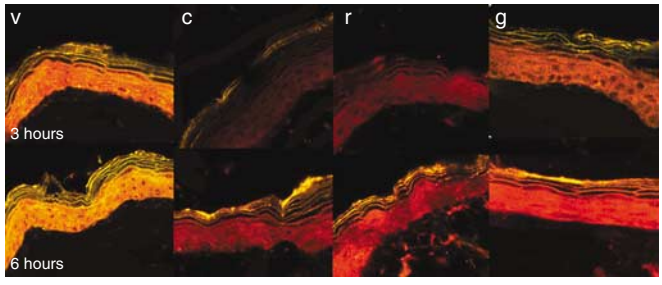

Figure 3. Broad-type and δ-specific PKC inhibitors impede the neutral lipid deposition during barrier recovery. Neutral lipid deposition in the epidermis was examined by Nile red staining. The upper line denotes 3 hours after barrier disruption and the lower line denotes 6 hours after barrier disruption. The vehicle-treated skin showed an intense red staining in the Malphigian layer and SC at 3 and 6 hours (v). The chelerythrine chloride-treated skin showed very weak staining in the epidermis at 3 hours and delayed staining at 6 hours (c). Similar weak and delayed staining was seen in rottlerin-treated skin (r). Gö6976-treated skin showed an intense red staining in the epidermis, similar to vehicle-treated skin (g).

in the chelerythrine chloride and rottlerin treatments, the number of LBs within the SG was significantly higher than in vehicle-treated skin (Table 1).

Effect of PKC inhibitors on epidermal calcium gradient

By using ultrastructural calcium capture cytochemistry, we observed changes in the epidermal calcium gradient. A progressive increase in the number of calcium precipitates, from the basal layers toward the SG, was observed in vehicle-treated skin (Figure 5a–c). A loss of the epidermal calcium gradient was observed at 3 hours after barrier disruption (Figure 5d). The topical application of the chelerythrine chloride resulted in increased calcium precipitates in the upper layer of the SG, though a nearly normal epidermal calcium gradient was observed at 3 hours after barrier disruption (Figure 5e). Similar findings were seen in the rottlerin-treated skin. Rottlerin-treated skin also showed an epidermal calcium gradient at 3 hours after barrier disruption (Figure 5, ROT-3 hours). Gö6976-treated skin showed a normal loss of calcium gradient at 3 hours, much like vehicle-treated skin (Figure 5, GO-3 hours). To determine whether the Ca^{2+} gradient was abrogated and then returned, or not abrogated at all after barrier disruption and rottlerin treatment, we observed a time course at 30 minutes and 1 hour. At 30 minutes, rottlerin-treated skin showed an abrogated epidermal calcium gradient. Although TEWL was still high and barrier recovery was not complete at 1 and 3 hours (Figure 2), the epidermal calcium gradient was rapidly reformed (Figure 5, ROT). In the case of Gö6976-treated skin, a well-formed epidermal calcium gradient was not seen at 30 minutes, 1, or 3 hours (Figure 5, GO).

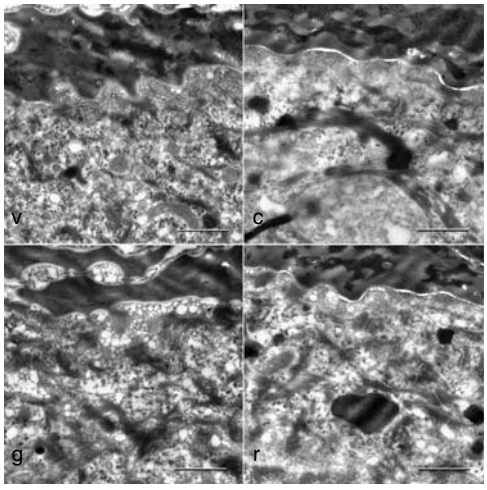

Figure 4. Broad-type and δ-specific PKC inhibitors impede the LB secretion. OsO_4 post-fixation was used for LB counting. Vehicle-treated skin showed that LBs were actively secreted to the SC and LBs within the SG were decreased or exhausted (v), as is normally seen in untreated skin. Although there was acute barrier disruption, electron micrographs showed that the secretion of LB between the SC and the SG was significantly decreased, and considerable numbers of LB remained in the upper layers of the SG in chelerythrine chloride-treated skin (c). Similar findings were seen in rottlerin-treated skin. Rottlerin-treated skin also showed a decrease in the number of LBs between the SC and SG. Many LBs remained in the upper layers of the SG 3 hours after barrier disruption (r). The PKC-α-specific inhibitor, Gö6976, treated skin showed normal secretion of LB to the SC and fewer LBs remained in the SG (g) (OsO_4, bar = 1 μm).

The effects of broad-type of PKC inhibitor on $[Ca^{2+}]_i$

To define the effect of PKC inhibitors *in vitro*, we examined the $[Ca^{2+}]_i$ level by using Fura-2. As shown in Figure 6a, in untreated control keratinocytes, the increase in extracellular free calcium level ($[Ca^{2+}]_o$) induced an increase in $[Ca^{2+}]_i$. The elevated $[Ca^{2+}]_i$ returned to baseline when $[Ca^{2+}]_o$ was decreased (Figure 6a). To test the effect of the PKC inhibitor, we treated keratinocytes with 5 μM chelerythrine chloride. In contrast to the untreated keratinocytes, chelerythrine chloride treatment caused a marked increase in $[Ca^{2+}]_i$ with increasing $[Ca^{2+}]_o$. Furthermore, the elevated $[Ca^{2+}]_i$ did not return to the baseline level after lowering $[Ca^{2+}]_o$ (Figure 6b). Chelerythrine chloride inhibited the decreasing $[Ca^{2+}]_i$ in response to an extracellular calcium change.

Effects of PKC isoenzyme-specific inhibitors on $[Ca^{2+}]_i$

To define the effect of PKC isoenzyme-specific inhibitors *in vitro*, we examined $[Ca^{2+}]_i$ levels by using Fura-2. To test the effect of the PKC-α-specific inhibitor, we treated keratinocytes with 0.5 mM Gö6976. As shown in Figure 6c, Gö6976-treated keratinocytes showed that an increase in $[Ca^{2+}]_o$ induced an increase in $[Ca^{2+}]_i$, which tended to return to baseline when $[Ca^{2+}]_o$ decreased (Figure 6a). In contrast to the untreated control and Gö6976-treated keratinocytes, rottlerin-treated keratinocytes showed a more marked increase in $[Ca^{2+}]_i$ when $[Ca^{2+}]_o$ increased, which did not

Table 1. Comparison of the number of LBs in the electron microscopic pictures

	Chelerythrine chloride-treated skin					Rottlerin-treated skin					Vehicle-treated skin					Chelerythrine *vs* vehicle	Rottlerin *vs* vehicle
Number of LBs between the SC and SG (secreted LB)	21	10	32	8	28	19	31	28	15	18	3	9	1	6	7	$P<0.05$	$P<0.05$
Total number (mean±SD)	97 (19.8±10.6)					133 (22.2±6.2)					26 (5.2±3.2)						
Number of LBs within the SG (unsecreted LB)	3	9	7	2	4	5	2	6	1	2	3	16	12	10	4	$P<0.05$	$P<0.05$
Total number (mean±SD)	25 (5.0±2.9)					16 (3.2±2.2)					55 (9.0±5.5)						

LB, lamellar body; SC, stratum corneum; SG, stratum granulosum.

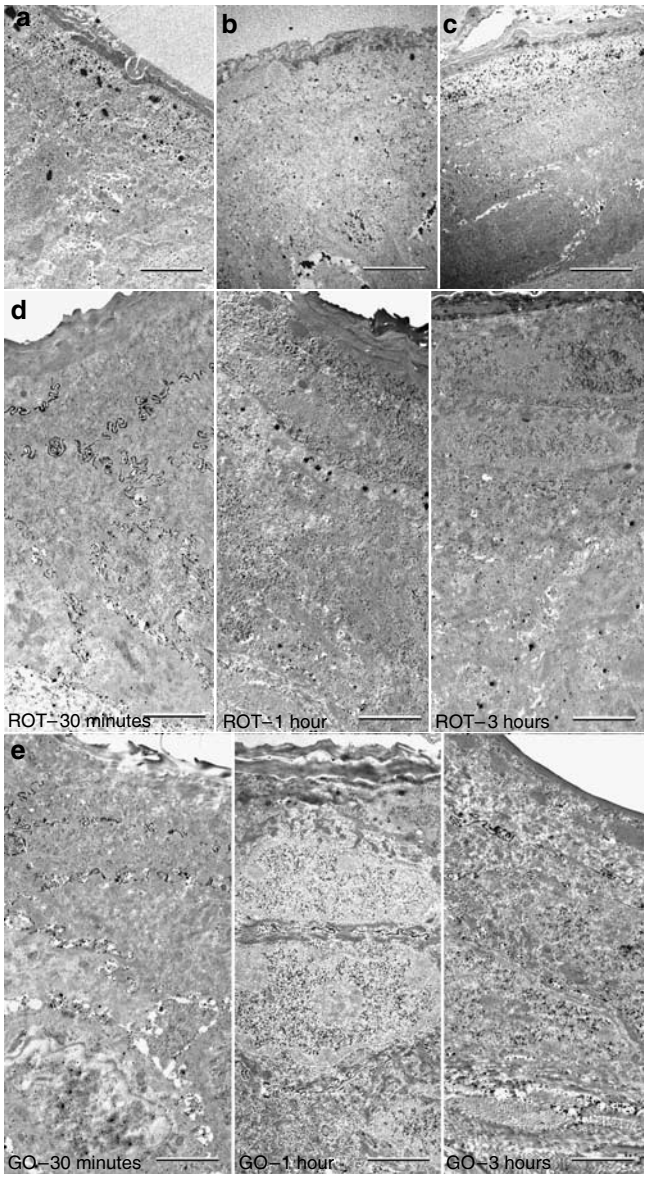

Figure 5. Although there was barrier disruption and incomplete barrier recovery, broad-type and δ-specific PKC inhibitor-treated skin showed the well-formed epidermal calcium gradient. (**a–c**) Visualization of epidermal calcium gradient by ultrastructural calcium capture cytochemistry shows a progressive increase in the number of calcium precipitates from the basal layers toward the SG in vehicle-treated skin. (**d**) A loss of the epidermal calcium gradient was observed at 3 hours after barrier disruption. (**e**) The topical application of the chelerythrine chloride resulted in an increase in calcium precipitates in the upper layer of SG, though a nearly normal epidermal calcium gradient was observed at 3 hours after barrier disruption (bar = 3 μm). In case of isoform-specific PKC inhibitors, we observed a time course at 30 minutes and 1 hour. At 30 minutes, the rottlerin-treated skin showed abrogated an epidermal calcium gradient, but the gradient was rapidly reformed at 1 and 3 hours (ROT 30 minutes, 1, 3 hours). In the case of Gö6976-treated skin, a well-formed epidermal calcium gradient was not seen at 30 minutes, 1 or 3 hours (GO 30 minutes, 1, 3 hours) (bar = 2 μm).

return to the baseline level when $[Ca^{2+}]_o$ was decreased (Figure 6). Among the several isoform-specific PKC inhibitors, rottlerin, a PKCδ-specific inhibitor, inhibited the $[Ca^{2+}]_i$ response to extracellular calcium change.

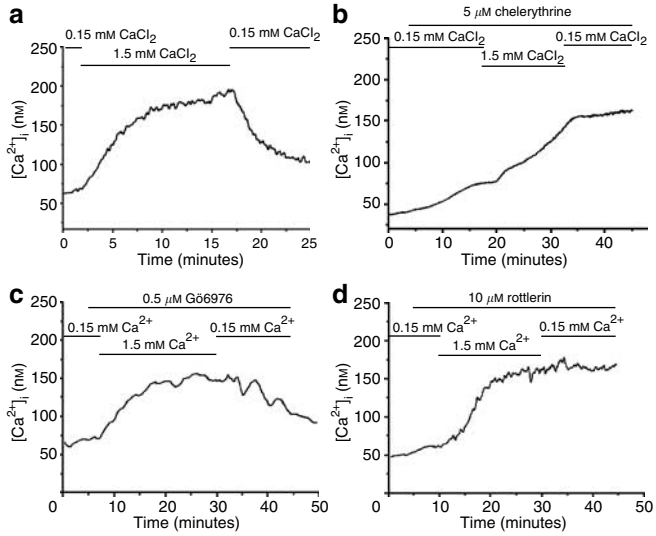

Figure 6. Broad-type and delta-specific PKC inhibitors impede the decrease of $[Ca^{2+}]_i$. An increase of $[Ca^{2+}]_o$, due to moving from 0.15 to 1.5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid-buffered Ca^{2+} solution, induced an increase in $[Ca^{2+}]_i$. (**a**) The elevated $[Ca^{2+}]_i$ returned to baseline with a reduction in $[Ca^{2+}]_o$ to 0.15 mM. In the case of chelerythrine chloride (5 μM)-treated keratinocytes, there was slight increase in the $[Ca^{2+}]_i$ baseline. (**b**) The $[Ca^{2+}]_o$ increase from 0.15 to 1.5 mM induced a $[Ca^{2+}]_i$ increase that did not return to baseline after decreasing the $[Ca^{2+}]_o$ to 0.15 mM. (**c**) Gö6976 (0.5 μM)-treated keratinocytes showed a $[Ca^{2+}]_i$ change similar to that seen in untreated keratinocytes. (**d**) In rottlerin (10 μM)-treated keratinocytes, $[Ca^{2+}]_i$ did not return to baseline and remained elevated, despite a decrease in $[Ca^{2+}]_o$ to 0.15 mM. The data in this graph represent the mean of 10 cells.

DISCUSSION

Several signals have been suggested in mediating the homeostatic processes that take place in cutaneous barrier disruption response. As barrier disruption is always accompanied by an increase in TEWL, it is reasonable to speculate that this water loss and the consequent change in keratinocyte tonicity induce the stimulation. The fact that occlusion can block the lipid and DNA synthesis responses to barrier disruption suggests that TEWL is also a regulatory signal in barrier homeostasis. But, since immediately exposing barrier-perturbed skin to isotonic, hypertonic, or hypotonic external solutions results in normal barrier recovery, water movement is not considered a major signal in barrier homeostatic responses (Lee *et al.*, 1992).

In addition to skin barrier homeostasis, calcium ions act as a major regulator in keratinocyte differentiation and proliferation. The epidermis has a calcium gradient, with the highest levels in the SG and lowest levels in the basal cell layer (Menon *et al.*, 1985a), which is very important for both permeability barrier homeostasis and epidermal differentiation. The Ca^{2+} gradient disappears after acute barrier disruption, and reappears in parallel with barrier restitution (Menon *et al.*, 1992a; Mao-Qiang *et al.*, 1997; Mauro *et al.*, 1998). In a previous study, it was shown that immersion of barrier-disrupted skin into a calcium ion-containing solution significantly delayed barrier recovery. In addition, if potassium and, to a lesser extent, magnesium and phosphorous

ions are present in the bathing solution, barrier recovery is also impeded. Moreover, calcium and potassium ions together appear to be synergistic in inhibiting barrier recovery (Lee *et al*., 1992). The central role of calcium ions in skin barrier homeostasis was demonstrated by the manipulation of LB secretion through changing the epidermal calcium gradient and without significantly changing TEWL, by iontophoresis or sonophoresis. These studies suggest that changes in calcium ion concentration in the SG can directly transduce the homeostatic signals for barrier repair, even without a change in permeability barrier function (Lee *et al*., 1998a).

In most of the cases, however, the role of the extracellular calcium gradient and the decrease in $[Ca^{2+}]_i$ in cutaneous barrier homeostasis were treated separately. Increased water movement, followed by passive calcium displacement outward, could explain the extracellular calcium ion loss from epidermis. Mauro *et al*. (1998) recorded changed calcium ion concentrations in the outer epidermis from 460 ± 57 to 128 ± 14 mg/kg immediately after barrier disruption. Most of the calcium ions contributing to this measurement are localized in the epidermal extracellular space. As there is a substantial and tightly controlled difference between intracellular free calcium ion and extracellular calcium ion concentrations, even with a significant decrease, the extracellular calcium level is still much higher than $[Ca^{2+}]_i$ (Mauro, 2003). The precise mechanism of $[Ca^{2+}]_i$ decrease in response to a loss of the extracellular calcium gradient is still not clear.

In this study, we investigated the effects of PKC inhibitors on barrier recovery. Collectively, our results can be explained as follows. Broad-type PKC inhibitors and a PKCδ-specific inhibitor reduced barrier recovery by inhibiting the intracellular Ca^{2+} response to loss of the extracellular Ca^{2+} gradient. While nearly all results were similar between these two inhibitors, as seen in Figure 6b and d, the calcium trace of chelerythrine chloride is somewhat different from that of rottlerin. The calcium trace of chelerythrine chloride-treated keratinocytes showed an unstable and increasing baseline. When the extracellular calcium level was increased, the calcium trace increase in chelerythrine chloride was slower than in rottlerin. As this type of calcium trace can be seen in an unregulated calcium influx such as a cytotoxic effect, we carried out a cytotoxicity study for chelerythrine chloride, rottlerin, and Gö6976 with alamar blue. This study showed less than 10% cytotoxicity in the chelerythrine chloride-treated cultured cells. Rottlerin and Gö6976 did not show any cytotoxicity at all. Although the chelerythrine chloride calcium trace showed an increasing baseline and slower $[Ca^{2+}]_i$ response, we believe these results are secondary effects of weak cytotoxicity, and mask the typical calcium curve (Figure 6d). As chelerythrine chloride is a broad-type PKC inhibitor, these results may be due to blocking by PKC isoenzymes other than δ. Regardless, the chelerythrine chloride results are not totally insignificant.

These results suggest that, among the PKC isoenzymes extant in keratinocytes, PKCδ may be associated with this phenomenon. It is also possible, however, that PKC inhibitors increase $[Ca^{2+}]_i$ not only by inhibiting the intracellular Ca^{2+} response to decreased $[Ca^{2+}]_o$, but also by affecting calcium influx through the calcium channel. Conversely, these findings indirectly suggest that PKC is involved in barrier recovery by keeping $[Ca^{2+}]_i$ elevated.

At this point, the role of PKC in keratinocyte differentiation is well known, but its role in barrier recovery response is not. Only Murata *et al*. (2000) were able to demonstrate that both serine-palmitoyl transferase and glucosylceramide synthetase expression are upregulated by a PKC-dependent mechanism in cultured human keratinocytes. Also, no reports on the mechanism explain the calcium elevation effect that takes place in keratinocytes when the barrier is disrupted. Similar $[Ca^{2+}]_i$ regulation is seen in other cells, namely granulosa cells. Basic fibroblast growth factor prevents granulosa cell apoptosis by decreasing $[Ca^{2+}]_i$ (Lynch *et al*., 2000). Peluso *et al*. (2001) elucidated the mechanism through which basic fibroblast growth factor-activated PKCδ regulates $[Ca^{2+}]_i$ within a physiological range by stimulating calcium efflux. They proposed that activated PKC functions to serine/threonine phosphorylate various molecular targets that may include calcium efflux regulators, such as plasma membrane calcium-adenosine triphosphatase. As has been shown in other cell types, D12-*O*-tetradecanoylphorbol-13-acetate-dependent phosphorylation of plasma membrane calcium-adenosine triphosphatase enhances the rate of calcium efflux, thereby maintaining calcium homeostasis (Garcia and Strehler, 1999). Studies have found, with results similar to ours, that the plasma membrane calcium-adenosine triphosphatase pump is localized to the plasma membrane and its activity is influenced by PKC (Blaustein and Lederer, 1999; Philipson and Nicoll, 2000; Therien and Blostein, 2000). PKCδ is the only PKC isotype that is expressed by granulosa cell. The existence of PKCα, δ, ε, η, and ζ in keratinocytes is well documented (Dlugosz *et al*., 1992; Fischer *et al*., 1993; Denning *et al*., 1995; Lee *et al*., 1998b), and other PKC isoforms (PKCβ, γ, and μ) have also been found in various keratinocytes (Fischer *et al*., 1993; Fisher *et al*., 1993; Rennecke *et al*., 1996). Among the normal keratinocyte PKC isoenzymes, we studied α, δ, and ζ because these are representative enzymes of conventional, novel, and atypical PKCs, respectively. PKCδ, the novel PKC isoform, is diacylglycerol sensitive but calcium insensitive. Therefore, we feel that PKCδ may be a more likely candidate for calcium regulation than isoenzymes subjected to skewed $[Ca^{2+}]$ following acute barrier disruption. In fact, our proposed mechanism is missing a link between the extracellular calcium decrease and PKC activation. A protein such as CaR, which functions in the case of elevated extracellular calcium level, could fill this gap. We found that there was an increase of diacylglycerol, an endogenous PKC activator, when the barrier is disrupted (data not shown), but we could not find a signal or receptor to link the decrease of extracellular calcium to diacylglycerol elevation. Our suggested mechanism of calcium regulation by PKC is shown in Figure 7. When the permeability barrier is disrupted, an increase in transepidermal water loss is followed by an extracellular calcium decrease in the outer epidermis. This extracellular calcium loss leads to the transduction of an

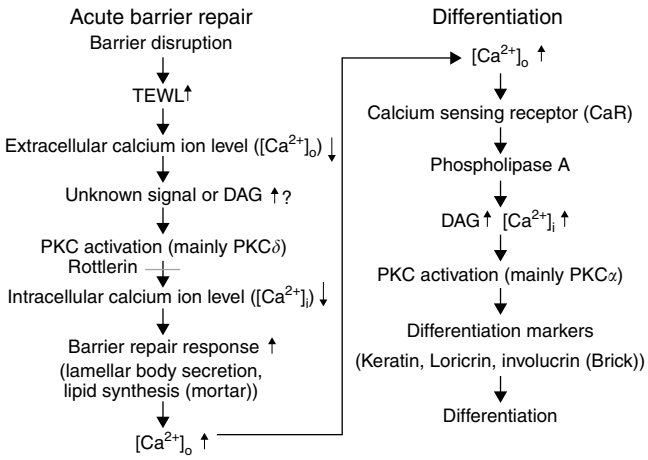

Figure 7. The suggested mechanism of intracellular calcium regulation by PKC.

unknown signal in keratinocytes. This signal may increase the level of diacylglycerol and activate PKC, which in turn regulates the $[Ca^{2+}]_i$ decrease by stimulating calcium efflux. Thus, a variety of responses for barrier repair are induced. Future studies will be needed to test this hypothesis.

MATERIALS AND METHODS

Animals

Male mice that were 8–12 weeks old and hairless were purchased from the animal laboratory of Yonsei University and used in this study. The Institutional Review Board at Yonsei University College of Medicine approved the use of mice, and the Medical Ethical Committee of the Yonsei University College of Medicine approved all described studies.

Reagents

The polyethylene glycol (Mw 300) and ethanol used as vehicles were purchased from Sigma Chemical (St Louis, MO). The broad-type PKC inhibitors, calphostin C and chelerythrine chloride, were purchased from Calbiochem (San Diego, CA). Other PKC isoenzyme-specific inhibitors were purchased from Biomol (Plymouth meeting, PA).

Barrier disruption

The epidermal permeability barrier of hairless male mice, 8–12 weeks old, was broken by tape stripping. The procedure was terminated when the TEWL reached 40 mg/cm^2/hour. A normal TEWL is less than 0.2 mg/cm^2/hour. TEWL was measured with a TM210 (Courage & Khazaka, Cologne, Germany). TEWL measurements were taken at the treatment sites 30 minutes, 1, 3, and 6 hours after barrier disruption. The barrier recovery results are expressed as percent recovery calculated by the following formula: (TEWL immediately after barrier disruption – TEWL at indicated time point)/(TEWL immediately after barrier disruption–baseline TEWL) × 100%.

Topical application of PKC inhibitor

Chelerythrine chloride and other isotype-specific inhibitors of PKC were dissolved in DW/polyethylene glycol/ethanol (1:3:1 vol/vol). In all, 10 μl of each inhibitor solution was applied to a 3 cm × 1 cm area of barrier disrupted mouse skin immediately after barrier disruption for 10 minutes. Neither the mixture nor the vehicle was irritating under the conditions of this experiment.

Electron microscopic examination

OsO_4 post-fixation. OsO_4 post-fixation was used to count the layers of SC and the number of LB, and RuO_4 post-fixation was used to observe LB secretion and the lipid structure of the SC intercellular space. Biopsy specimens were immediately cut into 1-mm^3 pieces and fixed in modified Karnovsky's fixative overnight, washed in 0.1 M cacodylate buffer, and post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 1 hour. After rinsing in buffer, samples were dehydrated in graded ethanol solutions, and embedded in an epon–epoxy mixture. Ultrathin sections (60–80 nm) were examined in a transmission electron microscope (H-7600, Hitachi, Japan) after further contrasting with uranyl acetate-lead citrate. We compared the specimens of chelerythrine chloride-treated skin with that of the control vehicle-treated skin at 30 minutes after barrier disruption. We took electron microscopy pictures from each specimen and the number of SC layers and LBs per picture (magnification × 25,000) were counted. We placed five 1 × 1 inch square grids at the junction of the SC and SG and counted within them to assess LB secretion. We placed the same grids at the SG for assessing the number of unsecreted LB.

Calcium-capture cytochemistry. Biopsy specimens were immediately cut into small pieces (0.5 mm^3) in a drop of ice-cold fixative (2% glutaraldehyde, 2% formaldehyde, 90 mM potassium oxalate, and 1.4% sucrose) and fixed overnight on ice (Menon *et al.*, 1985b). The fixative was removed and the samples were post-fixed in osmium tetroxide/potassium pyroantimonate for 2 hours on ice and washed for 10 minutes in ice-cold distilled water (pH 10). The samples were then dehydrated in a graded ethanol series, and embedded in epon–epoxy resin. Ultrathin sections (60–80 nm) were examined in a transmission electron microscope (H-7600, Hitachi, Japan). Each section was incubated with EDTA as a control.

Cell culture

Skin was obtained from newborn mice and treated with 0.5% trypsin in Hank's balanced salt solution solution (pH 7.4) overnight at 4°C. The epidermis was then mechanically separated from the dermis, and epidermal cells were released in Hank's balanced salt solution with 0.05% DNase and 20% fetal bovine serum. After washing, the keratinocytes were seeded in a collagen-coated dish at 1 × 10^6 cells with keratinocyte serum-free medium. The calcium concentration in the medium was adjusted by addition of 0.3 M $CaCl_2$ stock. Cells were cultured in medium with 0.05 mM calcium to maintain a proliferating population of basal cells. Terminal differentiation was induced by exposing the culture to medium containing 2 mM calcium for 24 hours.

Measurement of $[Ca^{2+}]_i$

Differentiated cultured hairless mouse keratinocytes were loaded with 4 μM Fura-2 AM (Kd = 224 nM) in 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer (130 mM sodium chloride, 3 mM potassium chloride, 2 mM magnesium chloride, 1 mg/ml pyruvate, 10 mM glucose, and 0.03 mM calcium chloride; pH 7.4) for 30 minutes. Cells were then washed and resuspended in buffer

twice. Fluorescence was recorded with a spectrofluorometer (Photon Technology International, Brunswick, NJ), coupled to a microscope (Nikon, Tokyo, Japan) using the fluorescence ratios at 340 and 380 nm (F340/F380) excitation and emission wavelengths, respectively. Each sample was calibrated by the addition of ionomycin (10 μM final concentration) (F_{max}), followed by 0.1% Triton X-100 and 10 mM EGTA/Tris (pH 8.3; F_{min}). $[Ca^{2+}]_i$ was calculated with the following formula:

$$[Ca^{2+}]_i = K_D \times S_{f2}/S_{b2}[(R - R_{min})/(R_{max} - R)]$$

where K_D is the dissociation constant of Fura-2; R the fluorescence ratio measured at $\lambda_{340/330}$; R_{min} the fluorescence ratio measured at PC12 cells after 2.5 mM EGTA treatment; R_{max} the fluorescence ratio measured at PC12 cells after 25 mM ionomycin treatment; S_{f2} the fluorescence intensity of λ_{380} at R_{min}; and S_{b2} the fluorescence intensity of λ_{380} at R_{max}.

Measurement of the cytotoxicity

In this experiment, cytotoxicity of the PKC inhibitors was measured with the alamar blue™ method. Human immortalized keratinocyte HaCaT cells were used. Cells were seeded in 96-well plates (1×104 cells/well). When the cells were 80% confluent, the media was removed and the cells were washed with Ca^{2+}, Mg^{2+}-free PBS (pH 7.4) solution. After washing, cells were added to each well and incubated in 175 μl of a tested inhibitor for 1 hour. After incubation, solutions were removed and cells were washed with PBS. Each well was incubated in 175 μl of alamar blue™ solution for 24 hours. After incubation, alamar blue™ reduction was determined by measuring absorbance at 570 and 600 nm with a microplate reader (Spectramax 340PC, Molecular Device, Sunnywale, CA). A standard curve was obtained for the alamar blue™ reduction by HaCaT cells and the number of viable cells was calculated.

CONFLICT OF INTEREST

The authors state no conflict of interest.

REFERENCES

Agner T (1992) Noninvasive measuring methods for the investigation of irritant patch test reactions. A study of patients with hand eczema, atopic dermatitis and controls. *Acta Dermatol Venereol Suppl* 173:1–26

Berardesca E, Fideli D, Borroni G, Rabbiosi G, Maibach H (1990) *In vivo* hydration and water-retention capacity of stratum corneum in clinically uninvolved skin in atopic and psoriatic patients. *Acta Dermatol Venereol* 70:400–4

Blaustein MP, Lederer WJ (1999) Sodium/calcium exchange: its physiological implications. *Physiol Rev* 79:763–854

Denning MF, Dlugosz AA, Williams EK, Szallasi Z, Blumberg PM, Yuspa SH (1995) Specific protein kinase C isozymes mediate the induction of keratinocyte differentiation markers by calcium. *Cell Growth Differ* 6: 149–57

Dlugosz AA, Mischak M, Mushinski JF, Yuspa SH (1992) Transcripts encoding protein kinase C alpha, delta, epsilon, zeta, and eta are expressed in basal and differentiating mouse keratinocytes *in vitro* and exhibit quantitative changes in neoplastic cell. *Mol Carcinogen* 5:286–92

Feingold KR (1997) Permeability barrier homeostasis. Its biochemical basis and regulation. *Cosmet Toilet* 112:49–59

Fischer SM, Lee ML, Maldve RE *et al.* (1993) Association of protein kinase C activation with ornithine decarboxylase in murine but not in human keratinocyte cultures. *Mol Carcinogen* 7:228–37

Fisher GJ, Tavakkol A, Leach K *et al.* (1993) Differential expression of protein kinase C isoenzymes in normal and psoriatic adult human skin: reduced expression of protein kinase C-betaII in psoriasis. *J Invest Dermatol* 101: 553–9

Garcia ML, Strehler EE (1999) Plasma membrane calcium ATPases as critical regulators of calcium homeostasis during neuronal cell function. *Front Biosci* 4:D869–82

Ghadially R, Reed JT, Elias PM. (1996) Stratum corneum structure and function correlates with phenotype in psoriasis. *J Invest Dermatol* 107:558–64

Hachem J, De Paepe K, Vanpee E, Kaufman L, Rogiers V, Roseeuw D (2001) Combination therapy improves the recovery of the skin barrier function: an experimental model using a contact allergy patch test combined with TEWL measurements. *Dermatology* 202:314–9

Held E, Lund H, Agner T (2001) Effect of different moisturizers on SLS-irritated human skin. *Contact Dermatitis* 44:229–34

Holleran WM, Feingold KR, Man MQ, Gao WN, Lee JM, Elias PM (1991) Regulation of epidermal sphingolipid synthesis by permeability barrier function. *J Lipid Res* 32:1151–8

Holleran WM, Takagi Y, Menon GK, Jackson SM, Lee JM, Feingold KR *et al.* (1994) Permeability barrier requirements regulate epidermal glucocerebrosidase. *J Lipid Res* 35:905

Lee SH, Choi EH, Feingold KR, Jiang S, Ahn SK (1998a) Iontophoresis itself on hairless mouse skin induces the loss of the epidermal calcium gradient without skin barrier impairment. *J Invest Dermatol* 111:39–43

Lee SH, Elias PM, Proksch E, Menon GK, Mao-Quiang M, Feingold KR (1992) Calcium and potassium are important regulators of barrier homeostasis in murine epidermis. *J Clin Invest* 89:530

Lee YS, Yuspa SH, Dlugosz AA (1998b) Differentiation of cultured human epidermal keratinocytes at high cell densities is mediated by endogenous activation of the protein kinase C pathway. *J Invest Dermatol* 111:762–6

Loden M, Olsson H, Axell T, Linde YW (1992) Friction, capacitance and transepidermal water loss (TEWL) in dry atopic and normal skin. *Br J Dermatol* 126:137–41

Lynch K, Fernandez G, Pappalardo A, Peluso JJ (2000) Basic fibroblast growth factor inhibits apoptosis of spontaneously immortalized granulosa cells by regulating intracellular free calcium levels through a protein kinase C delta dependent pathway. *Endocrinology* 141:4209–17

Mao-Qiang M, Mauro T, Bench G, Warren R, Elias PM, Feingold KR (1997) Calcium and potassium inhibit barrier recovery after disruption, independent of the type of insult in hairless mice. *Exp Dermatol* 6:36–40

Mauro T (2003) The discovery channel': CRAC'king the code of calcium influx. *J Invest Dermatol.* 121:IX–X

Mauro T, Bench G, Sidderas-Haddad E, Feingold K, Elias P, Cullander C (1998) Acute barrier perturbation abolishes the Ca2 and K gradients in murine epidermis: quantitative measurement using PIXE. *J Invest Dermatol* 111:1198–201

Menon GK, Elias PM, Lee SH, Feingold KR (1992a) Localization of calcium in murine epidermis following disruption and repair of the permeability barrier. *Cell Tissue Res* 270:503–12

Menon GK, Feingold KR, Elias PM (1992b) Lamellar body secretory response to barrier disruption. *J Invest Dermatol* 98:279–89

Menon GK, Feingold KR, Moser AH, Brown BE, Elias PM (1985a) *De novo* sterologenesis in the skin. II. Regulation by cutaneous barrier requirements. *J Lipid Res* 26:418–27

Menon GK, Grayson S, Elias PM (1985b) Ionic calcium reservoirs in mammalian epidermis: ultrastructural localization by ion-capture cytochemistry. *J Invest Dermatol* 84:508–12

Murata S, Uchida Y, Ichikawa S, Hirabayashi Y, Holleran WM (2000) Regularion of Glucosylceramide synthase expression in cultured Human Keratinocytes. *J Invest Dermatol* 114:795 (abstr.)

Peluso JJ, Pappalardo A, Fernandez G (2001) Basic fibroblast growth factor maintains calcium homeostasis and granulosa cell viability by stimulating calcium efflux via a PKC-dependent pathway. *Endocrinology* 142: 4203–11

Philipson KD, Nicoll DA (2000) Sodium–calcium exchanger: a molecular perspective. *Annu Rev Physiol* 62:111–33

Proksch E, Feingold KR, Man MQ, Elias PM (1991) Barrier function regulates epidermal DNA synthesis. *J Clin Invest* 87:1668–73

Rennecke J, Johannes FJ, Richter KH, Kittstein W, Marks F, Gschwendt M (1996) Immunological demonstration of protein kinase C μ in murine tissues and various cell lines. Differential recognition of phosphorylated forms and lack of down-regulation upon 12-*O*-tetradecanoylphorbol-13-acetate treatment of cells. *Eur J Biochem* 242:428–32

Suetake T, Sasai S, Zhen Y, Ohi T, Tagami H (1996) Functional analyses of the stratum corneum in scars. Sequential studies after injury and comparison among keloids, hypertrophic scars, and atrophic scars. *Arch Dermatol* 132:1453–8

Tagami H, Yoshikuni K (1985) Interrelationship between water barrier and reservoir functions of pathologic stratum corneum. *Arch Dermatol* 121:642–5

Therien AG, Blostein R (2000) Mechanisms of sodium pump regulation. *Am J Physiol Cell Physiol* 279:C541–66

Watanabe M, Tagami H, Horii I, Takahashi M, Kligman AM (1991) Functional analyses of the superficial stratum corneum in atopic xerosis. *Arch Dermatol* 127:1689–92

Werner Y, Lindberg M (1985) Transepidermal water loss in dry and clinically normal skin in patients with atopic dermatitis. *Acta Dermatol Venereol* 65:102–5